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Intestinal microbiota is affected by *Helicobacter pylori* infection in Japanese adolescents aged 14 or 15 years: a cross-sectional study

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6 **Title: Intestinal microbiota is affected by *Helicobacter pylori* infection in Japanese**
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9 **adolescents aged 14 or 15 years: a cross-sectional study**
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14 **Short title: Microbiota and *H. pylori* in adolescents**
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ABSTRACT

Objective: The relationship between *Helicobacter pylori* and the intestinal microbiota has not been clearly demonstrated in children and/or adolescents. The present study aimed to evaluate the effects of *H. pylori* infection on the intestinal microbiota in adolescents using genetic analysis.

Design: a cross-sectional study

Setting and participants: We included subjects from a longitudinal project involving *H. pylori* screening and treatment of junior high school third-grade students (aged 14 or 15 years) in Saga Prefecture. The study included a control group (n = 79) who were negative for anti-*H. pylori* antibody in urine and a *H. pylori* group (n = 80) who were positive for anti-*H. pylori* antibody in urine and *H. pylori* antigen in stool specimens.

Interventions: The intestinal microbiota was evaluated in stool specimens using 16S rRNA gene/DNA/amplicon sequencing with next generation sequencing.

Primary and secondary outcome measures: Alpha, beta diversity and relative abundances within the bacterial composition at the genus level in the control and *H. pylori* groups

Results: As shown by the alpha diversity of the 16S rRNA gene/DNA/amplicon sequence data, the control group had lower microbial species richness with lower alpha diversity compared with the *H. pylori* group ($P < 0.001$). Beta diversity of the intestinal microbiota profile also differed between the two groups ($P < 0.01$). The relative

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6 abundance of the *Prevotella* genus was higher in the *H. pylori* group ($P < 0.01$)
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9 concomitant with a gain in body mass index in the *H. pylori* group ($P < 0.01$) compared
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12 with the control group.

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14 **Conclusions:** The intestinal microbiota is significantly affected by *H. pylori* infection in
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17 Japanese adolescents. Additionally, the prevalence of the *Prevotella* genus is
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20 concomitantly increased along with the body mass index in *H. pylori*-infected students.

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23 **Trial registration number:** This study was registered with the University Hospital
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25
26 Medical Information Network (UMIN) Clinical Trials Registry (No. UMIN000028721).

27 28 **Strengths and limitations of this study**

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31 >The most strength of this study is that the effect of *Helicobacter pylori* (*H. pylori*)
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34 infection on the intestinal microbiota had been clearly demonstrated in children.
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36 > Because the participants were Japanese adolescents of almost the same age living in a
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39 single prefecture, it is presumed that there would be no major difference the two groups.
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42 >This study evaluated the intestinal microbiota using feces specimens, of which may be
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45 different from the mucosal-associated microbiota.
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48 >The effect of eradication of *H. pylori* on the intestinal microbiota could not be
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51 analyzed, because the eradication therapy is important for intestinal microbiota changes.

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55 **Keywords:** *Prevotella* genus, 16S rRNA, body mass index, screening and treatment
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6 **Abbreviations:** *Helicobacter pylori* = *H. pylori*, OTUs = operational taxonomic units,
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9 ANOVA = analysis of variance, PERMDISP = permutational analysis of multivariate
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12 dispersions
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14 **Word count:** 2,765 words
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INTRODUCTION

Newborns are exposed to various bacteria that are present in the mother's resident microbiota and the external environment. Bacterial species that comprise the intestinal microbiota change in an age-dependent manner^{1 2}. Development of the intestinal microbiota during infancy is affected by several factors, including the maternal resident microbiota^{3 4}, the method of nutrition for infants⁵⁻⁷, delivery style^{5 8 9}, and the administration of antibiotics^{3 10 11}.

Sustained infection of *Helicobacter pylori* decreases and/or increases gastric acid secretion, which might affect the gastric microbiota in adults¹²⁻¹⁴ and children^{12 13}. Several reports have suggested that the intestinal microbiota is significantly affected by *H. pylori* infection^{14 15}. The effect of *H. pylori* infection on the intestinal microbiota has been investigated in adults^{15 16}, but has not been clearly demonstrated in children.

Therefore, the present study aimed to examine junior high school students in Japan aged from 14 to 15 years to determine whether *H. pylori* infection changes the intestinal microbiota. We also examined how body mass index (BMI) affects the intestinal microbiota, in addition to *H. pylori* infection.

METHODS

2.1. Study design and subjects

The longitudinal project for *H. pylori* screening and treatment among junior high school third-grade students in Saga Prefecture started in 2016 with the aim of primary

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6 prevention of stomach cancer¹⁷. Figure 1 shows a flowchart of the junior high school
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9 third-grade students in Saga Prefecture in 2017. Among 8519 junior high school
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12 students aged 14 or 15 years old, 7230 received a screening urinary test (RAPIRAN;
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14 Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) to detect anti-*H. pylori*
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17 immunoglobulin-G antibody by immunochromatography. The diagnostic sensitivity,
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20 specificity, negative predictive value, and positive predictive value of the urinary test
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23 have been reported to be 78.4%, 100%, 90.1%, and 100%, respectively¹⁸. A total of
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26 6874 students tested negative for *H. pylori* with the urinary test and 79 of these students
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28
29 were randomly selected as the *H. pylori*-negative group (control group). Students who
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32 tested positive in the screening urinary test received an *H. pylori* stool antigen detection
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35 test (TESTMATE RAPID PYLORI ANTIGEN; Wakamoto Pharmaceutical Co., Ltd.
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38 Tokyo, Japan). Among 290 students who received the stool antigen test, 234 students
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41 were positive for *H. pylori* infection. Finally, 80 of these students were randomly
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44 selected as the *H. pylori*-positive group (*H. pylori* group). The exclusion criteria for the
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47 present study were as follows: i) students who had taken medications, including
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50 proton-pump inhibitors, H₂ receptor antagonists, antacids, probiotics, mucosal
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53 protective agents, and/or antibiotics within the 6 months prior to enrollment, ii) students
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56 who were in the outpatient hospital because of sickness, and iii) students who had
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59 undergone eradication therapy for *H. pylori*.
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6 The microbiota distribution was compared between the control and *H. pylori*
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9 groups regarding alpha diversity, beta diversity, and the relative abundance of the
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12 intestinal microbiota. The effect of BMI (low: < 15, middle: 15 to 25, high: > 25) on the
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15 microbiota distribution in the two groups was examined.
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20 **2.2. Stool sample collection and bacterial DNA extraction from feces**

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22 Each participant collected a stool sample at home for the present study using a paper
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24 stool collector and tube that was pre-filled with 5 ml of stool DNA stabilizer. Samples
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26 were immediately stored at -20°C and then delivered to the project center within 1 day.
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29 Extraction of bacterial DNA was performed as described previously¹⁹. A total of 20 mg
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32 of feces were washed three times in 1.0 ml of PBS and centrifuged (14,000 × g). The
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35 pellets were resuspended in a solution containing 450 µl of extraction buffer (100 mM
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37 Tris-HCl, 40 mM EDTA; pH 9.0) and 50 µl of 10% sodium dodecyl sulfate. A total of
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40 300 mg of glass beads (diameter, 0.1 mm) and 500 µl of buffer-saturated phenol were
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43 added to the suspension and vortexed vigorously. After centrifugation at 14,000 × g for
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46 5 min, 400 µl of the supernatant was extracted by phenol–chloroform, and 250 µl of the
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49 supernatant was subjected to isopropanol precipitation. Finally, the DNA was
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52 suspended in 1.0 ml of Tris-EDTA buffer.
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58 **2.3. DNA sequence analysis**

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6 Meta-analysis of the bacterial 16S rDNA sequences in the feces was performed in
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8 accordance with a previously described method²⁰ with minor modifications. In brief,
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10 the V3–V4 region of 16S rDNA was amplified on a Veriti thermal cycler (Thermo
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12 Fisher Scientific, Waltham, MA, USA). The amplicon was purified using AMPure XP
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14 magnetic beads (Beckman Coulter, Brea, CA, USA). For multiplex sequencing, a
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16 polymerase chain reaction was performed with dual eight-base indices (Nextera XT
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18 Index kit, Illumina, CA, USA). After purification by AMPure XP beads, the purified
19
20 barcoded library was quantified fluorometrically using a QuantiT PicoGreen ds DNA
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22 Assay Kit (Invitrogen, Paisley, UK) and pooled at the same volume. The library pool
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24 (10 pM) was spiked with 40% PhiX control DNA (10 pM). Sequencing was conducted
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26 on a MiSeq platform with MiSeq Reagent Kit v2 chemistry (Illumina).
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39 **2.4. Microbiota analysis**

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41 Removal of low-quality sequences and chimera sequences, construction of operational
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43 taxonomic units (OTUs), and taxonomy assignment were conducted using the
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45 Quantitative Insights Into Microbial Ecology pipeline (<http://qiime.org/>)²¹. In brief,
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47 50,000 raw reads were randomly obtained from the sequence files for each sample and
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49 merged by fastq-join with the default setting. Consequently, sequence reads with an
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51 average quality value of < 25 were removed and then chimera-checked. Five thousand
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53 reliable sequence reads were randomly obtained for each sample and OTUs were
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6 constructed by clustering with a 97% identity threshold. The representative reads of
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8 each OTU were then assigned to the 16S rRNA gene database using UCLUST with \geq
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10 97% identity²². Comparison of each taxon in the gut microbiota was conducted at the
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12 genus level. Beta diversity was estimated by computing the weighted and unweighted
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14 UniFrac distances between samples²³. To compare the differences in the overall
15
16 bacterial gut microbiota structure, principal co-ordinates analysis was applied to reduce
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18 the dimensionality of the resulting distance matrix. The Shannon index, observed OTUs,
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20 chao 1, and the abundance-based coverage estimator index were calculated to
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22 investigate the alpha diversity of the microbiota in the samples.
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33 **2.5. Statistical analysis**

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35 All statistical analyses were conducted with R statistical software (R Core Team (2018).
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37 R: A language and environment for statistical computing. R Foundation for Statistical
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39 Computing, Vienna, Austria. URL <https://www.R-project.org/>). Data are shown as
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41 mean \pm SE. Statistical significance was set at $P < 0.05$. During the analyses of the gut
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43 microbiotas, statistical significance was determined by a Welch's t test with Benjamini–
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45 Hochberg correlation. The relative abundance data were non-normally distributed;
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47 however, we applied Welch's T-test because the Mann–Whitney U-test is reported to be
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49 less robust²⁴. Beta diversity was analyzed using permutational analysis of multivariate
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51 dispersions (PERMDISP) for comparisons of gene similarity.
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RESULTS

3.1. Student characteristics

A total of 159 students participated in this study. The students' characteristics are shown in Table 1. There were no significant differences in sex, age, BMI, birth delivery style, method of infant nutrition, or the prevalence of allergic disease between the groups. The ratio of nursery school graduates to kindergarten graduates was significantly higher in the *H. pylori* group than in the control group ($P < 0.001$).

3.2. Alpha and beta diversity in the control and *H. pylori* groups

Figure 2 shows the alpha diversity of the 16S rRNA gene/DNA/amplicon sequence data. The control group showed lower microbial species richness with lower alpha diversity compared with the *H. pylori* group. The observed species index, chao 1 index, and ACE index all showed significantly higher diversity in the *H. pylori* group compared with the control group ($P < 0.001$). The Shannon index was not significantly different between the two groups ($P = 0.054$).

Figure 3 shows the beta diversity of the 16S rRNA gene/DNA/amplicon sequence data. Two-dimensional principle coordinate analysis of the weighted and unweighted UniFrac distances of the 16S rRNA gene/DNA/amplicon sequence data showed that the majority of samples were clustered dependent on the *H. pylori* infection status. The

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6 analysis of similarity showed that the differences were significant for the weighted
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9 UniFrac distance ($P < 0.001$), but no significant for the unweighted UniFrac distance (P
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12 = 0.643) using PERMDISP.

13 14 15 16 17 **3.3. Relative abundances within the bacterial composition at the genus level for the** 18 19 20 **two groups**

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23 Figure 4 shows the 13 main types of bacteria present in the intestinal microbiota at the
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25 genus level as follows: *Bacteroides*, *Blautia*, *Bifidobacterium*, *Faecalibacterium*,
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27 *Prevotella*, *Fusicatenibacter*, *Eubacterium*, *Anaerostipes*, *Subdoligranulum*,
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29 *Streptococcus*, *Megamonas*, *Collinsella*, and *Clostridium*. The relative abundances of
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31 the *Prevotella* genus ($P < 0.01$) and *Collinsella* genus ($P < 0.05$) were significantly
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33 higher in the *H. pylori* group than in the control group. The relative abundance of the
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35 *Subdoligranulum* genus was significantly higher in the control group than in the *H.*
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37 *pylori* group ($P < 0.01$).
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51 52 53 **3.4. BMI and the relative abundances within the bacterial composition at the genus** 54 55 56 **level**

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58 In the control and *H. pylori* groups, the intestinal microbiota was evaluated in
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60 association with BMI. Figure 5 shows the seven main types of bacteria in the intestinal
microbiota at the genus level for the control group and the *H. pylori* group, categorized

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6 by BMI. For the control group, these included *Bacteroides*, *Blautia*, *Bifidobacterium*,
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9 *Prevotella*, *Faecalibacterium*, *Fusicatenibacter*, and *Megamonas*. For the *H. pylori*
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11 group, these included *Prevotella*, *Bacteroides*, *Blautia*, *Bifidobacterium*,
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14 *Faecalibacterium*, *Megamonas*, and *Fusicatenibacter*. In the *H. pylori* group, the
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16 relative abundance of the *Prevotella* genus was significantly higher in the high BMI
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18 group compared with the middle and low BMI groups (both $P < 0.01$). Furthermore, the
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20 relative abundance of the *Prevotella* genus in the middle BMI group was higher than
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22 that in the low BMI group ($P < 0.05$). The relative abundances of *Bacteroides* and
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24 *Bifidobacterium* were significantly lower in the high BMI group compared with the
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26 other two groups (both $P < 0.05$). In the *H. pylori* group, BMI had no effect on the
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28 relative abundances of *Blautia*, *Faecalibacterium*, *Megamonas*, and *Fusicatenibacter*.
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31 In the control group, the relative abundance of the *Prevotella* genus was not
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33 significantly higher in the high BMI group compared with the middle and low BMI
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35 groups, whereas the relative abundance of the *Prevotella* genus significantly increased
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37 in proportion to an increase in BMI in the *H. pylori* group (low BMI vs high BMI: $P <$
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39 0.001, middle BMI vs high BMI: $P < 0.001$) (Figure 6). The *Subdoligranulum* genus
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41 had a lower relative abundance in the high BMI category than in the low BMI group,
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43 but this trend was observed not only in the *H. pylori* group but also in the control group
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45 (Figure 7). The *Collinsella* genus was not associated with BMI regardless of *H. pylori*
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47 infection status (Figure 8).
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DISCUSSION

The present study revealed two clinically important results: i) *H. pylori* infection significantly affected the intestinal microbiota of adolescents aged 14 or 15 years, as determined for Japanese junior high school students; ii) An increase in the relative abundance of the *Prevotella* genus in *H. pylori*-infected adolescents was concomitant with a gain in BMI.

Most reports of the effects of *H. pylori* on the intestinal microbiota based on the analysis of feces samples were in adults and data were lacking for children^{25 26}. The present study showed a difference in the intestinal microbiota between *H. pylori*-infected and non-infected adolescents based on feces specimens. Alpha diversity, bacterial richness, and variance all showed greater diversity in *H. pylori*-infected students than in controls (Figure 2). A previous study showed that the diversity of the gastric microbiota in adolescents was enhanced by *H. pylori* infection¹². Studies of the relationship between the intestinal microbiota and *H. pylori* infection are limited. One study reported a decrease in the *Firmicutes* genus in the human duodenal mucosa during *H. pylori* infection²⁷. In the *H. pylori* infection model of Mongolian gerbils, the abundances of the *Bacteroides* and *Enterococcus* genera were increased in the duodenal mucosa²⁸. In adults, the intestinal microbiota has been shown to be reduced in diversity during *H. pylori* infection¹⁶ and our results were similar to those reported in adults

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6 previously (Figure 2, 3). The human gut microbiota has been reported to form by the
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8 age of 3 years ²⁹, so it may be that there is no difference in the effects of *H. pylori*
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10 infection on the intestinal microbiota between adolescents and adults.
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14 It is known that infection with *H. pylori* reduces gastric acid secretion in children
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16 ^{30 31}. It was further suggested that a decrease in gastric acid secretion due to *H. pylori*
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18 infection may affect the intestinal flora of adolescents with *H. pylori* infection. In
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20 addition, a decrease in gastric acid secretion caused by *H. pylori* infection may allow a
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22 wide variety of bacteria in the oral cavity to more easily pass through the stomach and
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24 reach the lower gastrointestinal tract, thereby affecting the intestinal flora in feces. This
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26 might explain the result of the present study that alpha diversity of the fecal intestinal
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28 microbiota was increased in students with *H. pylori* infection. As suggested by the
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30 present study, *H. pylori* infection might be a factor that disturbs the intestinal
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32 microbiota in adolescents. The mechanisms and clinical importance of the effect of *H.*
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34 *pylori* warrant further investigation.
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44 The *Prevotella* genus increased in abundance during *H. pylori* infection, and
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46 this increase was found to be concomitant with a rise in BMI in the present study. A
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48 previous report indicated that the *Prevotella* genus was elevated in abundance in
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50 school-age children infected with *H. pylori* ³². The *Bacteroides* and *Bifidobacterium*
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52 genera are dominant among the intestinal microbiota in Japanese children ³³. A previous
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54 study showed that the prevalence rate of the *Prevotella* genus in the intestinal
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6 microbiota was higher in subjects who consumed carbohydrates more frequently ³⁴,
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9 which suggests that the *Prevotella* genus is closely related to eating habits. A
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11 correlation between *H. pylori* infection and the onset of diabetes has been reported in
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13 epidemiology studies ^{35 36}, but the reason for this remains unknown. Meanwhile, the
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15 prevalence of the *Prevotella* genus increased in patients with obesity ^{37 38}, nonalcoholic
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17 steatohepatitis ³⁹, hyperlipidemia ⁴⁰, and even in gestational diabetes, which is
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19 considered as a diabetes mellitus preliminary group ⁴¹. The *Prevotella* genus is
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21 considered to contribute to hyperglycemia and insulin resistance ^{38 42 43}. In the present
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23 study, an increase in the relative abundance of *Prevotella* genus was observed in *H.*
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25 *pylori*-infected children with an increased BMI (Figure 5, 6). *H. pylori* infection in
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27 children with an elevated BMI without diabetes mellitus, caused an increase in the
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29 prevalence of the *Prevotella* genus (Figure 5, 6) and, as a result, insulin resistance
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31 increased, which may predispose individuals to diabetes mellitus. In fact, it is thought
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33 that the increase in *Prevotella* genus may be involved in the process of developing
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35 abnormal glucose metabolism as a result of obesity ^{44 45}.
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47 The *Subdoligranulum* genus showed a lower relative abundance in the high
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49 BMI category than in the low BMI group, but this trend was seen not only in the *H.*
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51 *pylori* group but also in the control group (Figure 7). The *Collinsella* genus was not
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53 associated with BMI regardless of *H. pylori* infection status (Figure 8). It has been
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55 reported that the *Subdoligranulum* genus is less prevalent among type 2 diabetes
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6 patients compared with their non-diabetic counterparts ⁴⁶, and a negative correlation
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9 with insulin resistance has been shown ⁴⁷. It has been reported that an increase in
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11 *Collinsella* genus is associated with an increase in insulin, triglyceride, and very low
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13 density lipoprotein levels ⁴⁸, and is associated with type 2 diabetes ⁴⁹. In our study, of
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15 the three genera (*Prevotella*, *Subdoligranulum*, and *Collinsella*) that showed significant
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17 differences in relative abundance between the *H. pylori* and control groups, the
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19 *Prevotella* genus showed the most significant correlation between *H. pylori* infection
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21 status and BMI. The *Prevotella* genus was the only genus that showed an association
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23 with BMI in the *H. pylori* group but not the control group.
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31 There are several limitations to the present study. i) The present study
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33 evaluated feces specimens, the microbiota of which may be different from the
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35 mucosal-associated microbiota. ii) The effect of eradication of *H. pylori* on the
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37 intestinal microbiota could be important ⁵⁰, and we plan to investigate this in the future.
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39 iii) There was a difference in preschool status between the two groups (Table 1), and it
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41 could not be completely ruled out that this could have affected the intestinal microbiota.
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50 CONCLUSION

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52 The present study shows that the intestinal microbiota is significantly affected by *H.*
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54 *pylori* infection in junior high school third-grade students in Saga Prefecture, Japan.
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6 Furthermore, the relative abundance of the *Prevotella* genus was increased
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9 concomitantly with a rise in BMI in *H. pylori*-infected students.
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15
16
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22
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28 **Author contributors**

29
30
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32
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34
35 contribution did not influence the analysis or interpretation of the data in this study. The
36
37 authors (YT and HO) did not play any additional role in the study design, data
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22 participants included in the study. Signed informed consent was obtained from each
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24 study participant prior to participation in the study.
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33 institutional review board of Saga University Hospital (approval number: 2016-11-03).
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35 Written informed consent was obtained from all of the students and their guardians. All
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37 methods were carried out in accordance with relevant guidelines
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39 and regulations or Helsinki guidelines.
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47 **Provenance and peer review:** Not commissioned; externally peer-reviewed.
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53 **Date availability statement:** The datasets used and/or analyzed during the current
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55 study are available from the corresponding author on reasonable request.
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Table 1. Background characteristics of junior high school students in the two groups

		Control group (n = 79)	<i>H. pylori</i> group (n = 80)	<i>P</i> value
Sex	(male/female)	42/37	46/34	0.80
Age	(years)	14.73 ± 0.33	14.76 ± 0.32	0.71
BMI	(kg/m ²)	19.69 ± 3.48	19.67 ± 2.41	0.97
Delivery	(vaginal/C-section)	68/11	60/11	0.79
Nutrition	(breast/formula/mix)	37/6/36	27/15/36	0.07
School	(nursery/kindergarten/none)	25/54/0	53/25/2	< 0.001
Allergies	(+/-)	5/75	7/73	0.55

Delivery: birth delivery style; C-section: cesarean section; nutrition: method of infant nutrition; school: pre-school situation; BMI: body mass index.

Figure legends

Figure 1. Flowchart for *Helicobacter pylori* screening and treatment of junior high school students in Saga Prefecture and the selection method used to obtain the two groups.

The *H. pylori* group comprised 80 students who tested positive for urinary anti-*H. pylori* immunoglobulin-G antibody, gave a positive stool antigen test, and consented to the study. The control group (n = 79) comprised those who tested negative for both tests.

Figure 2. Alpha diversity of the 16S rRNA sequences in the control and *H. pylori* groups

The control group showed lower microbial species richness compared with the *H. pylori* group. The observed species index (S. obs), chao 1 index, and abundance-based coverage estimator index all showed significantly higher diversity in the *H. pylori* group than in the control group ($*P < 0.001$). The Shannon index was not significantly different between the two groups ($P = 0.054$). OTUs: operational taxonomic units.

Figure 3. Beta diversity of the 16S rRNA/DNA/amplicon sequence data (control group vs *H. pylori* group)

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6 PCO: principal coordinate analysis; PERMDISP: permutational analysis of multivariate
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14 **Figure 4. The main 13 types of bacteria present in the intestinal microbiota at the**
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25 **Figure 5. The seven main types of bacteria present in the intestinal microbiota at**
26 **the genus level for the control group (A) and the *H. pylori* group (B) in association**
27 **with body mass index (BMI)**
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33 * $P < 0.05$; ** $P < 0.01$. Low: BMI < 15; Mid: BMI of 15 to 25; High: BMI > 25.
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39 **Figure 6. Relative abundance of the *Prevotella* genus in relation to BMI category in**
40 **the *H. pylori* and control groups**
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53 **Figure 7. Relative abundance of the *Subdoligranulum* genus in relation to BMI**
54 **category in the *H. pylori* and control groups**
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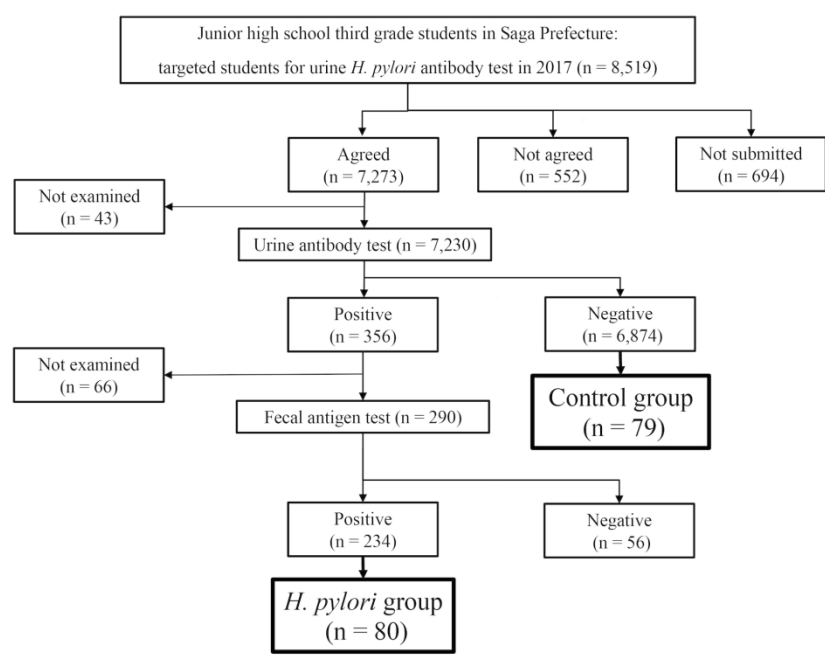
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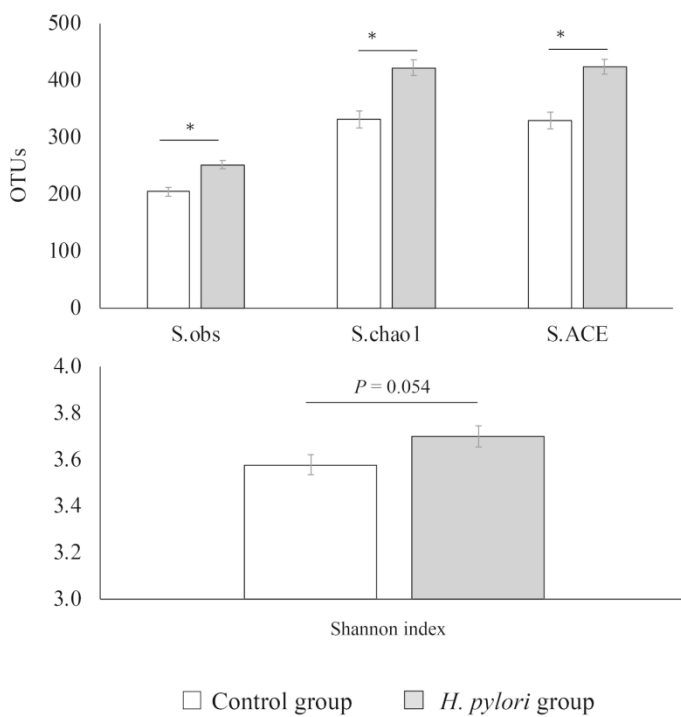
11 **Figure 8. Relative abundance of the *Collinsella* genus in relation to BMI category**
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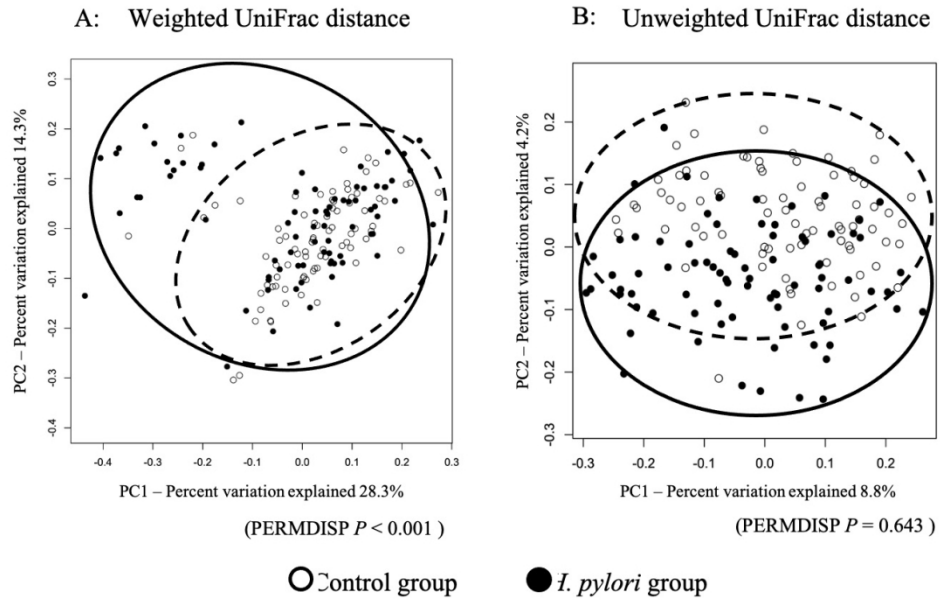


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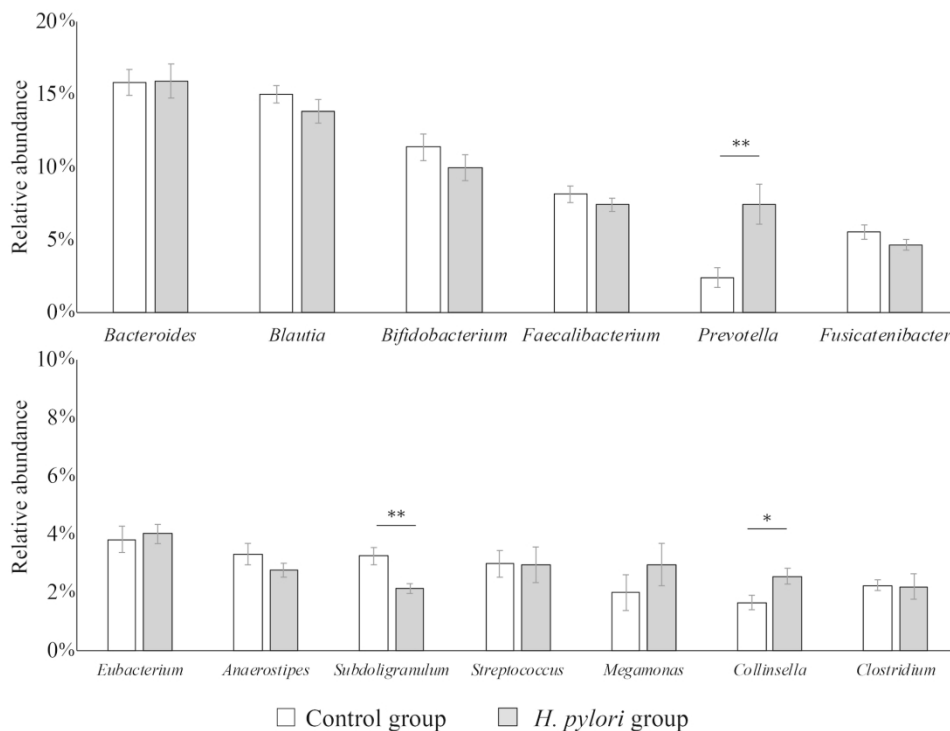


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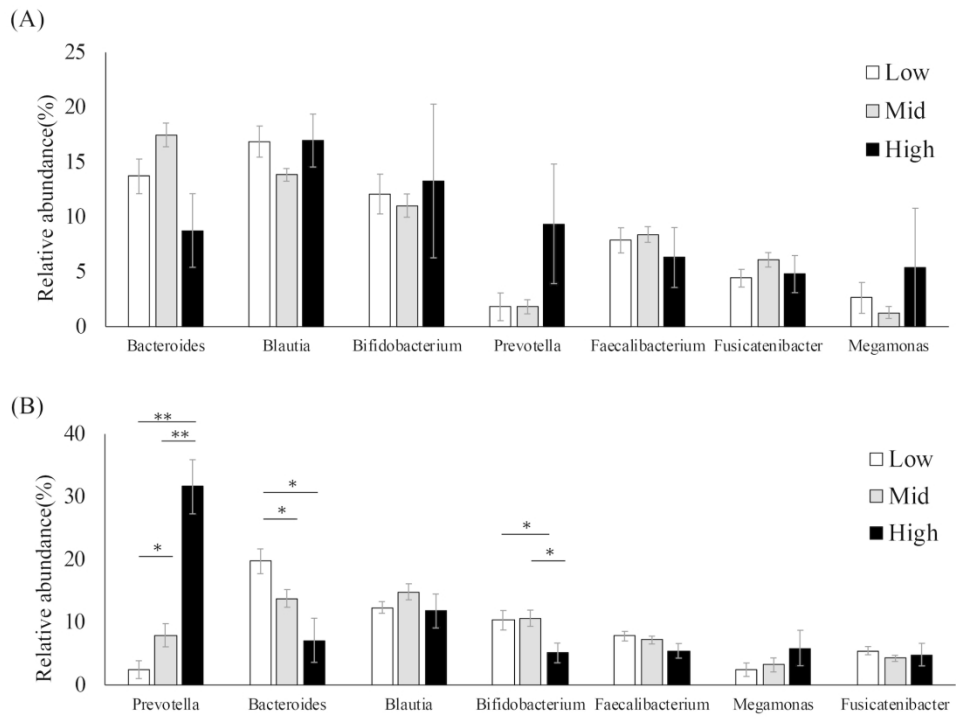


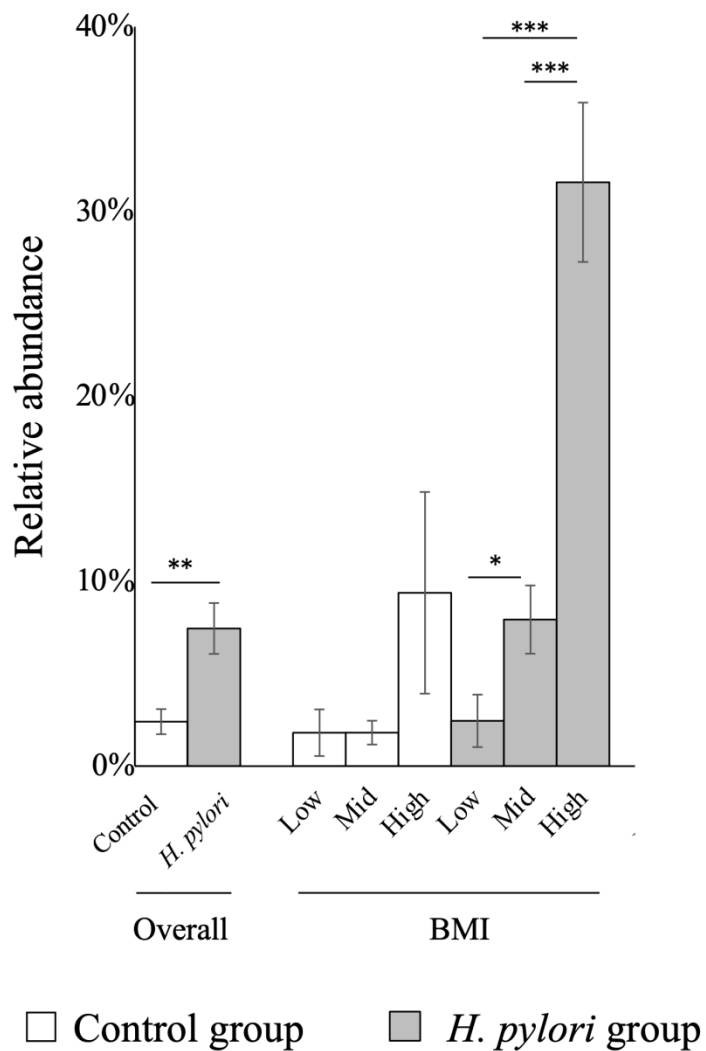
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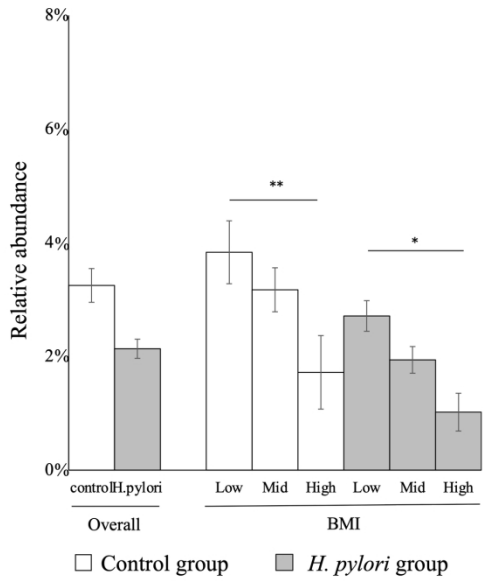
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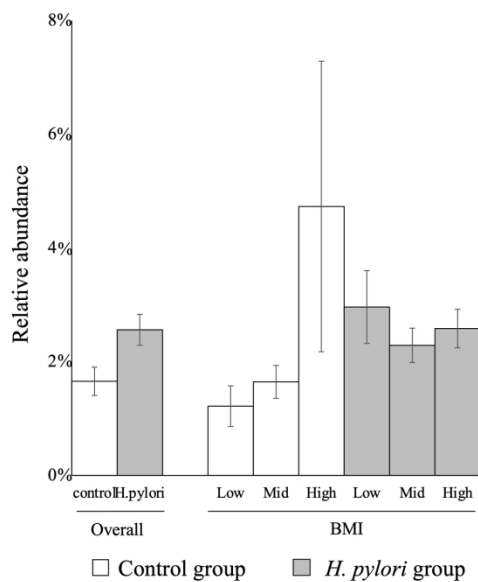


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			Page Number
Title and abstract			
Title	#1a	Indicate the study's design with a commonly used term in the title or the abstract	1
Abstract	#1b	Provide in the abstract an informative and balanced summary of what was done and what was found	2-3
Introduction			
Background / rationale	#2	Explain the scientific background and rationale for the investigation being reported	5
Objectives	#3	State specific objectives, including any prespecified hypotheses	5
Methods			
Study design	#4	Present key elements of study design early in the paper	5-6
Setting	#5	Describe the setting, locations, and relevant dates, including periods of	5-6

		recruitment, exposure, follow-up, and data collection	
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2	Eligibility criteria	#6a Give the eligibility criteria, and the sources and methods of selection of participants.	6
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6		#7 Clearly define all outcomes, exposures, predictors, potential	6-9
7		confounders, and effect modifiers. Give diagnostic criteria, if applicable	
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10	Data sources /	#8 For each variable of interest give sources of data and details of methods	6-9
11	measurement	of assessment (measurement). Describe comparability of assessment	
12		methods if there is more than one group. Give information separately	
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16	Bias	#9 Describe any efforts to address potential sources of bias	6-9
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19	Study size	#10 Explain how the study size was arrived at	6-9
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21	Quantitative	#11 Explain how quantitative variables were handled in the analyses. If	6-9
22	variables	applicable, describe which groupings were chosen, and why	
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25	Statistical	#12a Describe all statistical methods, including those used to control for	9
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29	Statistical	#12b Describe any methods used to examine subgroups and interactions	9
30	methods		
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33	Statistical	#12c Explain how missing data were addressed	9
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37	Statistical	#12d If applicable, describe analytical methods taking account of sampling	9
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41	Statistical	#12e Describe any sensitivity analyses	9
42	methods		
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44	Results		
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46	Participants	#13a Report numbers of individuals at each stage of study—eg numbers	10
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55	Participants	#13b Give reasons for non-participation at each stage	10
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57	Participants	#13c Consider use of a flow diagram	10
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1	Descriptive data	#14a	Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders. Give information separately for exposed and unexposed groups if applicable.	10
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6	Descriptive data	#14b	Indicate number of participants with missing data for each variable of interest	10
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10	Outcome data	#15	Report numbers of outcome events or summary measures. Give information separately for exposed and unexposed groups if applicable.	10-12
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14	Main results	#16a	Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	10-12
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19	Main results	#16b	Report category boundaries when continuous variables were categorized	10-12
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21	Main results	#16c	If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	10-12
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25	Other analyses	#17	Report other analyses done—e.g., analyses of subgroups and interactions, and sensitivity analyses	10-12
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29	Discussion			
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31	Key results	#18	Summarise key results with reference to study objectives	13
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34	Limitations	#19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias.	16
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39	Interpretation	#20	Give a cautious overall interpretation considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.	13-16
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44	Generalisability	#21	Discuss the generalisability (external validity) of the study results	13-16
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47	Other			
48	Information			
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51	Funding	#22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	18
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Helicobacter pylori infection-induced changes in the intestinal microbiota of 14 or 15 year-old Japanese adolescents: A cross-sectional study

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1 **Title: *Helicobacter pylori* infection-induced changes in the intestinal microbiota of**
2 **14 or 15 year-old Japanese adolescents: A cross-sectional study**

3
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17 **ABSTRACT**

18 **Objective:** The relationship between *Helicobacter pylori* and the intestinal microbiota
19 has not yet been clearly demonstrated in children and adolescents. The present study
20 aimed at evaluating how *H. pylori* infection could affect the intestinal microbiota in
21 adolescents using genetic analysis.

22 **Design:** cross-sectional study

23 **Setting and participants:** We included subjects from a longitudinal project involving
24 *H. pylori* screening and treatment of junior high school third-grade students (aged 14 or
25 15 years) in Saga Prefecture. The study included a control group (n = 79) and an
26 *H. pylori* group (n = 80) tested negative and positive for the anti-*H. pylori* antibody in
27 the urine and *H. pylori* antigen in stool specimens, respectively.

28 **Interventions:** The intestinal microbiota was evaluated in stool specimens using 16S
29 rRNA gene/DNA/amplicon sequencing with next-generation sequencing.

30 **Primary and secondary outcome measures:** We assessed alpha and beta diversity,
31 just as well as relative abundances within the bacterial composition at the genus level in
32 both groups.

33 **Results:** As shown by the alpha diversity of the 16S rRNA gene/DNA/amplicon
34 sequence data, the control group exhibited lower microbial species richness with lower

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6 35 alpha diversity compared with the *H. pylori* group ($P < 0.001$). The beta diversity of the
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9 36 intestinal microbiota profile also differed between the two groups ($P < 0.01$). The
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12 37 relative abundance of the *Prevotella* genus was higher in the *H. pylori* group ($P < 0.01$)
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15 38 concomitant with a gain in body mass index in the *H. pylori* group ($P < 0.01$) compared
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18 39 with the control group.

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21 40 **Conclusions:** *H. pylori* infection significantly affected the intestinal microbiota in
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24 41 Japanese adolescents. In addition, the prevalence of the *Prevotella* genus is
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27 42 concomitantly increased along with the body mass index in *H. pylori*-infected students.

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30 43 **Trial registration number:** This study was registered in the University Hospital
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33 44 Medical Information Network (UMIN) Clinical Trials Registry (No. UMIN000028721).

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36 45 **Strengths and limitations of this study**

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39 46 • The most significant strength of this study is that it clearly demonstrated the effect
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42 47 of *Helicobacter pylori* (*H. pylori*) infection on the intestinal microbiota of children.
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45 48 • As the participants were Japanese adolescents of almost the same age living in a
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48 49 single prefecture, no major difference would presumably exist between the two
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51 50 groups.
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54 51 • This study evaluated the intestinal microbiota using feces specimens, which might
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57 52 differ from the mucosal-associated microbiota
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7 53 • The effect of *H. pylori* eradication on the intestinal microbiota could not be
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9 54 analyzed, as the eradication therapy is important for intestinal microbiota changes.
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15 56 **Keywords:** *Prevotella* genus, 16S rRNA, body mass index, screening and treatment
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21 58 **Abbreviations:** *Helicobacter pylori* = *H. pylori*, proton-pump inhibitors = PPIs, OTUs
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24 59 = operational taxonomic units, ANOVA = analysis of variance, PERMDISP =
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27 60 permutational analysis of multivariate dispersions, F/B ration = ratio of *Firmicutes*
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30 61 phylum to *Bacteroides* phylum
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62 INTRODUCTION

63 Newborns are exposed to various bacteria that are present in the mother's resident
64 microbiota and the external environment. Bacterial species that comprise the intestinal
65 microbiota change in an age-dependent manner [1, 2]. The development of the intestinal
66 microbiota during infancy is affected by several factors, including the maternal resident
67 microbiota [3, 4], the method of nutrition for infants [5-7], delivery style [5, 8, 9], and
68 the administration of antibiotics [3, 10, 11].

69 The global *H. pylori* prevalence in children varies significantly, from 2.5% in Japan
70 to 34.6% in Ethiopia [12]. Sustained infection of *Helicobacter pylori* decreases or
71 increases gastric acid secretion, which might affect the gastric microbiota in adults
72 [13-15] and children [13, 14]. Several previous reports have suggested that the intestinal
73 microbiota is significantly affected by *H. pylori* infection [15, 16]. The effect of *H.*
74 *pylori* infection on the intestinal microbiota has been demonstrated in adults [16, 17] but
75 has not been fully investigated in children.

76 Therefore, the present study aimed at examining junior high school students in
77 Japan aged 14–15 years to determine whether *H. pylori* infection changes the intestinal
78 microbiota. Moreover, we also examined how body mass index (BMI) affects the
79 intestinal microbiota, in addition to *H. pylori* infection.

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81 METHODS**82 2.1. Study design and subjects**

83 The longitudinal project for *H. pylori* screening and treatment among junior high school
84 third-grade students in Saga Prefecture started in 2016 with the aim of primary
85 prevention of stomach cancer [17]. Figure 1 shows a flowchart of the junior high school
86 third-grade students in Saga Prefecture in 2017. Among 8519 junior high school
87 students aged 14 or 15 years, 7230 received a screening urinary test (RAPIRAN; Otsuka
88 Pharmaceutical Co., Ltd., Tokyo, Japan) to detect anti-*H. pylori* immunoglobulin-G
89 antibody by immunochromatography. There is an established screening program for
90 kidney diseases in Saga Prefecture, targeting third-grade students in junior high schools.
91 Given the full inclusivity of student during this test through simple urine examination,
92 we used the established system to obtain urine samples to screen for *H. pylori* infection
93 [17]. The diagnostic sensitivity, specificity, negative predictive value, and positive
94 predictive value of the urinary test was reportedly 78.4, 100, 90.1, and 100%,
95 respectively [18]. A total of 6874 students tested negative for *H. pylori* with the urinary
96 test and 79 of these students were randomly selected as the *H. pylori*-negative group
97 (control group). Students who tested positive in the screening urinary test received an

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6 98 *H. pylori* stool antigen detection test (TESTMATE RAPID PYLORI ANTIGEN;
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9 99 Wakamoto Pharmaceutical Co., Ltd. Tokyo, Japan). Among 290 students who received
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12 100 the stool antigen test, 234 students tested positive for *H. pylori* infection. Finally, 80 of
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15 101 these students were randomly selected as the *H. pylori*-positive group (*H. pylori* group).
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18 102 The exclusion criteria for the present study were as follows: i) students who had taken
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21 103 medications, including proton-pump inhibitors (PPIs), H₂ receptor antagonists, antacids,
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24 104 probiotics, mucosal protective agents, and antibiotics within the 6 months prior to
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27 105 enrollment, ii) students who were in the outpatient hospital because of sickness, and iii)
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30 106 students who had undergone eradication therapy for *H. pylori*.

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33 107 The microbiota distribution was compared between the control and *H. pylori*
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36 108 groups regarding alpha diversity, beta diversity, and the relative abundance of the
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39 109 intestinal microbiota. The effect of BMI (low: <15, middle: 15–25, high: >25) on the
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42 110 microbiota distribution in the two groups was examined.
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46 112 **2.2. Stool sample collection and bacterial DNA extraction from feces**

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49 113 Each participant collected a stool sample at home for the present study using a paper
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52 114 stool collector and tube that was pre-filled with 5 ml of a stool DNA stabilizer. The
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57 115 stool collection method was performed according to the attached document of the stool
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6 116 collection kit. Samples were immediately stored at -20°C and delivered to the project
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9 117 center within a day. Extraction of bacterial DNA was performed as described previously
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12 118 [19]. A total of 20 mg of feces was washed three times in 1.0 ml of PBS and centrifuged
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15 119 ($14,000 \times g$). The pellets were resuspended in a solution containing 450 μl of extraction
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18 120 buffer (100 mM Tris-HCl, 40 mM EDTA; pH 9.0) and 50 μl of 10% sodium dodecyl
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21 121 sulfate. A total of 300 mg of glass beads (diameter, 0.1 mm) and 500 μl of
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24 122 buffer-saturated phenol were added to the suspension and vortexed vigorously. After
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27 123 centrifugation at $14,000 \times g$ for 5 min, 400 μl of the supernatant was extracted by
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30 124 phenol-chloroform, and 250 μl of the supernatant was subjected to isopropanol
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33 125 precipitation. Finally, the DNA was suspended in 1.0 ml of Tris-EDTA buffer.
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39 127 **2.3. DNA sequence analysis**

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42 128 We performed the meta-analysis of the bacterial 16S rDNA sequences in the feces in
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45 129 accordance with a previously described method [20] with minor modifications. Briefly,
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48 130 the V3–V4 region of 16S rDNA were amplified on a Veriti thermal cycler (Thermo
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51 131 Fisher Scientific, Waltham, MA, USA). The amplicon was purified using AMPure XP
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54 132 magnetic beads (Beckman Coulter, Brea, CA, USA). For multiplex sequencing, a
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57 133 polymerase chain reaction was performed with dual eight-base indices (Nextera XT
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6 134 Index kit, Illumina, CA, USA). After purification by AMPure XP beads, the purified
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9 135 barcoded library was quantified fluorometrically using a QuantiT PicoGreen ds DNA
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12 136 Assay Kit (Invitrogen, Paisley, UK) and pooled at the same volume. The library pool
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15 137 (10 pM) was spiked with 40% PhiX control DNA (10 pM). Sequencing was conducted
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18 138 on a MiSeq platform with MiSeq Reagent Kit v2 chemistry (Illumina).

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22 140 **2.4. Microbiota analysis**

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27 141 We conducted the removal of low-quality and chimera sequences, construction of
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30 142 operational taxonomic units (OTUs), and taxonomy assignment using the Quantitative
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33 143 Insights Into Microbial Ecology pipeline (<http://qiime.org/>) [21]. Briefly, 50,000 raw
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36 144 reads were randomly obtained from the sequence files for each sample and merged by
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39 145 fastq-join with the default setting. Consequently, sequence reads with an average quality
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42 146 value of <25 were removed and then chimera-checked. Five thousand reliable sequence
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45 147 reads were randomly obtained for each sample and OTUs were constructed by
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48 148 clustering with a 97% identity threshold. The representative reads of each OTU were
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51 149 then assigned to the 16S rRNA gene database using UCLUST with $\geq 97\%$ identity [22].
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54 150 A comparison of each taxon in the gut microbiota was conducted at the genus level.
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57 151 Beta diversity was estimated by computing the weighted and unweighted UniFrac
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6 152 distances between the samples [23]. In order to compare the differences in the overall
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9 153 bacterial gut microbiota structure, principal coordinates analysis was applied to reduce
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12 154 the dimensionality of the resulting distance matrix. We calculated the Shannon index,
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15 155 observed OTUs, chao 1, and the abundance-based coverage estimator index to
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18 156 investigate the alpha diversity of the microbiota in the samples.
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24 158 **2.5. Statistical analysis**

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27 159 All statistical analyses were conducted with the R statistical software (R Core Team
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30 160 (2018). R: A language and environment for statistical computing. R Foundation for
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33 161 Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>). Data are
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36 162 shown as the mean \pm SE. Statistical significance was set at $P < 0.05$. During the
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39 163 analyses of the gut microbiotas, the statistical significance was determined by Welch's
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42 164 t-test with Benjamini–Hochberg correlation. The relative abundance data were
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45 165 non-normally distributed. However, we applied Welch's t-test as the Mann–Whitney
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48 166 U-test is reportedly less robust [24]. Beta diversity was analyzed using permutational
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51 167 analysis of multivariate dispersions (PERMDISP) for comparisons of gene similarity.
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57 169 **2.6. Patient and Public Involvement**

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6 170 This study was performed without patient involvement. Patients were not invited to
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9 171 comment on the study design and were not consulted to develop patient-relevant
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12 172 outcomes or interpret the results. Patients were not invited to contribute to the writing or
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15 173 editing of the manuscript for readability or accuracy.
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21 175 **RESULTS**

24 176 **3.1. Student characteristics**

27 177 A total of 159 students participated in this study. The student characteristics are shown
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30 178 in Table 1. No significant differences could be observed in sex, age, BMI, birth delivery
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33 179 style, method of infant nutrition, or the prevalence of allergic disease between the
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36 180 groups. The ratio of nursery school graduates to kindergarten graduates was
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39 181 significantly higher in the *H. pylori* group than in the control group ($P < 0.001$). The
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42 182 subjects of this study did not include low-birth-weight infants (birth weight 2500 g or
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45 183 less). In addition, we did not investigate whether symptoms associated with *H. pylori*
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48 184 infection, such as abdominal symptoms, were present in the *H. pylori* group.
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54 186 **3.2. Alpha and beta diversity in the control and the *H. pylori* groups**

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6 187 Figure 2 shows the alpha diversity of the 16S rRNA gene/DNA/amplicon sequence data.
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9 188 The control group showed lower microbial species richness with lower alpha diversity
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12 189 compared with the *H. pylori* group. The observed species index, chao 1 index, and ACE
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15 190 index all showed significantly higher diversity in the *H. pylori* group compared with the
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18 191 control group ($P < 0.001$). The Shannon index was not significantly different between
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21 192 the two groups ($P = 0.054$).
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24 193 Figure 3 shows the beta diversity of the 16S rRNA gene/DNA/amplicon sequence
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27 194 data. The two-dimensional principal coordinate analysis of the weighted and
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30 195 unweighted UniFrac distances of the 16S rRNA gene/DNA/amplicon sequence data
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33 196 showed that the majority of samples were clustered dependent on the *H. pylori* infection
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36 197 status. The similarity analysis showed that the differences were significant for the
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39 198 weighted UniFrac distance ($P < 0.001$), but not for the unweighted UniFrac distance (P
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42 199 = 0.643) using PERMDISP.
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48 201 **3.3. Relative abundances within the bacterial composition at the genus level for the**
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51 202 **two groups**
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54 203 Figure 4 shows the 13 main bacterial types present in the intestinal microbiota at the
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57 204 genus level as follows: *Bacteroides*, *Blautia*, *Bifidobacterium*, *Faecalibacterium*,

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6 205 *Prevotella*, *Fusicatenibacter*, *Eubacterium*, *Anaerostipes*, *Subdoligranulum*,
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9 206 *Streptococcus*, *Megamonas*, *Collinsella*, and *Clostridium*. The relative abundances of
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12 207 the *Prevotella* genus ($P < 0.01$) and *Collinsella* genus ($P < 0.05$) were significantly
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15 208 higher in the *H. pylori* group than in the control group. The relative abundance of the
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18 209 *Subdoligranulum* genus was significantly higher in the control group than in the *H.*
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21 210 *pylori* group ($P < 0.01$). At the phylum level, the ratio of the *Firmicutes* to the
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24 211 *Bacteroides* phyla (F/B ratio) showed no significant difference between the two groups
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27 212 (the control group; 4.19 ± 3.27 vs. the *H. pylori* group; 4.87 ± 12.04 , $P = 0.63$).
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32 33 214 **3.4. BMI and the relative abundances within the bacterial composition at the genus** 34 35 36 215 **level** 37

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39 216 In the control and *H. pylori* groups, the intestinal microbiota was evaluated in
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42 217 association with the BMI. Figure 5 shows the seven main bacterial types in the
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45 218 intestinal microbiota at the genus level for the control and the *H. pylori* groups,
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48 219 categorized by the BMI. For the control group, these included *Bacteroides*, *Blautia*,
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51 220 *Bifidobacterium*, *Prevotella*, *Faecalibacterium*, *Fusicatenibacter*, and *Megamonas* and
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54 221 for the *H. pylori* group *Prevotella*, *Bacteroides*, *Blautia*, *Bifidobacterium*,
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57 222 *Faecalibacterium*, *Megamonas*, and *Fusicatenibacter*. In the *H. pylori* group, the
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6 223 relative abundance of the *Prevotella* genus was significantly higher in the high-BMI
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9 224 group compared with the middle- and low-BMI groups (both $P < 0.01$). Furthermore,
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12 225 the relative abundance of the *Prevotella* genus in the middle-BMI group was higher
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15 226 than that in the low-BMI group ($P < 0.05$). The relative abundances of *Bacteroides* and
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18 227 *Bifidobacterium* were significantly lower in the high-BMI group compared with the
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21 228 other two groups (both $P < 0.05$). In the *H. pylori* group, the BMI did not affect the
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24 229 relative abundances of *Blautia*, *Faecalibacterium*, *Magamonas*, and *Fusicatenibacter*.
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27 230 In the control group, the relative abundance of the *Prevotella* genus was not
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30 231 significantly higher in the high-BMI group compared with the middle- and low-BMI
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33 232 groups, whereas the relative abundance of the *Prevotella* genus significantly and
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36 233 proportionately increased with an increasing BMI in the *H. pylori* group (low BMI vs
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39 234 high BMI: $P < 0.001$, middle BMI vs high BMI: $P < 0.001$) (Figure 6). At the phylum
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42 235 level, we observed no significant differences in the F/B ratio among the three BMI
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45 236 categories in the control groups. However, a significant difference could be detected
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48 237 between the high and middle BMI categories in the *H. pylori* group (Figure 7). The
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51 238 *Subdoligranulum* genus had a lower relative abundance in the high-BMI category than
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54 239 in the low-BMI group, although this trend was observed not only in the *H. pylori* group
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6 240 but also in the control group (Figure 8). The *Collinsella* genus was not associated with
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9 241 the BMI regardless of *H. pylori* infection status (Figure 9).
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15 243 **DISCUSSION**

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18 244 The present study revealed two clinically important results: i) *H. pylori* infection
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21 245 significantly affected the intestinal microbiota of adolescents aged 14 or 15 years, as
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24 246 determined for Japanese junior high school students; ii) An increase in the relative
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27 247 abundance of the *Prevotella* genus in *H. pylori*-infected adolescents was concomitant
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30 248 with a gain in BMI.
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33 249 Most reports of the effects of *H. pylori* on the intestinal microbiota based on the
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36 250 analysis of feces samples were in adults and data were lacking for children [25, 26]. The
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39 251 present study showed a difference in the intestinal microbiota between *H.*
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42 252 *pylori*-infected and non-infected adolescents based on feces specimens. Alpha diversity,
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45 253 bacterial richness, and variance all showed greater diversity in *H. pylori*-infected
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48 254 students than in controls (Figure 2). A previous study showed that the diversity of the
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51 255 gastric microbiota in adolescents was enhanced by *H. pylori* infection [13]. Studies of
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54 256 the relationship between the intestinal microbiota and *H. pylori* infection are limited.
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57 257 One study reported a decrease in the *Firmicutes* genus in the human duodenal mucosa
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6 258 during *H. pylori* infection [27]. In the *H. pylori* infection model of Mongolian gerbils,
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9 259 the abundances of the *Bacteroides* and *Enterococcus* genera were increased in the
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12 260 duodenal mucosa [28]. In adults, *H. pylori* infection [17] reportedly reduced intestinal
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15 261 microbiota diversity and our results were in good agreement with these previous reports
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18 262 (Figure 2, 3). The human gut microbiota has been reported to form by the age of 3 years
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21 263 [29], so it may be that there is no difference in the effects of *H. pylori* infection on the
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24 264 intestinal microbiota between adolescents and adults.

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27 265 It is known that infection with *H. pylori* reduces gastric acid secretion in children
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30 266 [30, 31]. It was further suggested that a decrease in gastric acid secretion due to *H.*
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33 267 *pylori* infection may affect the intestinal flora of adolescents with *H. pylori* infection. In
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36 268 addition, a decrease in gastric acid secretion caused by *H. pylori* infection may allow a
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39 269 wide variety of bacteria in the oral cavity to more easily pass through the stomach and
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42 270 reach the lower gastrointestinal tract, thereby affecting the intestinal flora in feces. The
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45 271 inhibitory effect of PPIs on gastric acid secretion affects the composition of the
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48 272 intestinal flora. PPIs administration causes an increase in the indigenous bacteria the
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51 273 *Streptococcus* genus and the *Lactobacillus* genus in the intestine, which is thought to be
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54 274 due to the oral bacteria reaching the intestine to suppress gastric acid secretion [32, 33].
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57 275 This might explain the result of the present study that alpha diversity of the fecal
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6 276 intestinal microbiota was increased in students with *H. pylori* infection. As suggested by
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9 277 the present study, *H. pylori* infection might be a factor that disturbs the intestinal
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12 278 microbiota in adolescents. *H. pylori* infection is involved in the alterations of gut
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15 279 microbiota composition and diversity, which can lead to changes in production level
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18 280 and physiologic regulation of the gut metabolic hormones released from the host
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21 281 endocrine system [34]. The mechanisms and clinical importance of the effect of *H.*
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24 282 *pylori* warrant further investigation.

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27 283 The *Prevotella* genus increased in abundance during *H. pylori* infection, and
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30 284 this increase was found to be concomitant with a rise in BMI in the present study. A
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33 285 previous report indicated that the *Prevotella* genus was elevated in abundance in
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36 286 school-age children infected with *H. pylori* [35]. This was an epidemiological study,
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39 287 and unfortunately, it is completely unknown why at this time the *Prevotella* genus is
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42 288 elevated in school-age children infected with *H. pylori*. The *Bacteroides* and
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45 289 *Bifidobacterium* genera are dominant among the intestinal microbiota in Japanese
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48 290 children [36]. A previous study showed that the prevalence rate of the *Prevotella* genus
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51 291 in the intestinal microbiota was higher in subjects who consumed carbohydrates more
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54 292 frequently [37], which suggests that the *Prevotella* genus is closely related to eating
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57 293 habits. In the present study, it is not possible to determine whether infection with *H.*

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6 294 *pylori* affected the diet and resulted in an increase in the *Prevotella* genus, or whether
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9 295 infection with *H. pylori* increased the *Prevotella* genus and affected the diet, and caused
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12 296 an increase in BMI. In general, the F/B ratio has been found to increase with obesity
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15 297 [38]. The *Prevotella* genus belongs to the *Bacteroides* phylum, the present study thus
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18 298 exhibited inconsistency. At the moment, it is currently difficult to associate the F/B
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21 299 ratio with a determined health status and, more specifically, to consider it as a hallmark
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24 300 of obesity [39]. In the future, regarding the relationship between *H. pylori* and the
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27 301 *Prevotella* genus and BMI, it is necessary to analyze the intestinal flora in early
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30 302 childhood, including the history of eating habits from early childhood.

33 303 A correlation between *H. pylori* infection and the onset of diabetes has been
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36 304 reported in epidemiology studies [40, 41], but the reason for this remains unknown.
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39 305 Meanwhile, the prevalence of the *Prevotella* genus increased in patients with obesity
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42 306 [42, 43], nonalcoholic steatohepatitis [44], hyperlipidemia [45], and even in gestational
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45 307 diabetes, which is considered as a diabetes mellitus preliminary group [46]. The
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48 308 *Prevotella* genus is considered to contribute to hyperglycemia and insulin resistance [43,
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51 309 47, 48]. In the present study, an increase in the relative abundance of the *Prevotella*
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54 310 genus was observed in *H. pylori*-infected children with an increased BMI (Figure 5, 6).
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57 311 *H. pylori* infection in children with an elevated BMI without diabetes mellitus, caused
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6 312 an increase in the prevalence of the *Prevotella* genus (Figure 5, 6) and, as a result,
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9 313 insulin resistance increased, which may predispose individuals to diabetes mellitus. In
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12 314 fact, it is thought that the increase in *Prevotella* genus may be involved in the process of
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15 315 developing abnormal glucose metabolism as a result of obesity [49, 50].
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18 316 The *Subdoligranulum* genus showed a lower relative abundance in the high
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21 317 BMI category than in the low BMI group, but this trend was seen not only in the *H.*
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24 318 *pylori* group but also in the control group (Figure 7). The *Collinsella* genus was not
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27 319 associated with BMI regardless of *H. pylori* infection status (Figure 8). It has been
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29
30 320 reported that the *Subdoligranulum* genus is less prevalent among type 2 diabetes
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33 321 patients compared with their non-diabetic counterparts [51], and a negative correlation
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36 322 with insulin resistance has been shown [52]. An increase in the *Collinsella* genus is
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39 323 reportedly associated with increased insulin, triglyceride, and very-low-density
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42 324 lipoprotein levels [53] and is associated with type 2 diabetes [54]. In our study, of the
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45 325 three genera (*Prevotella*, *Subdoligranulum*, and *Collinsella*) that showed significant
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48 326 differences in relative abundance between the *H. pylori* and control groups, the
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51 327 *Prevotella* genus showed the most significant correlation between *H. pylori* infection
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54 328 status and BMI. The *Prevotella* genus was the only genus that showed an association
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57 329 with BMI in the *H. pylori* group but not the control group.
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6 330 There are several limitations to the present study. i) In the selection of subjects
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9 331 in both groups, false-negative results by using the urinary antibody in the control group
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12 332 and false-positive results by using stool antigen in the *H. pylori* group could not be
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15 333 completely eliminated. ii) The present study evaluated feces specimens, the microbiota
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18 334 of which may be different from the mucosal-associated microbiota. iii) The effect of
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21 335 eradication of *H. pylori* on the intestinal microbiota could be important [55], and we
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24 336 plan to investigate this in the future. iv) There was a difference in preschool status
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27 337 between the two groups (Table 1), and it could not be completely ruled out that this
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30 338 could have affected the intestinal microbiota.

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340 **CONCLUSION**

341 The present study shows that the intestinal microbiota is significantly affected by *H.*
342 *pylori* infection in junior high school third-grade students in Saga Prefecture, Japan.
343 Furthermore, the relative abundance of the *Prevotella* genus was increased
344 concomitantly with a rise in BMI in *H. pylori*-infected students.

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33 356 **Author contributors**

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39 358 performing intestinal microbiota analysis and statistical evaluation. However, their
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42 359 contribution did not influence data analysis or interpretation in this study. The authors
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45 360 (YT and HO) did not play any additional role in the study design, data collection and
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48 361 analysis, publishing decisions, or manuscript preparation.
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51 362 Study concept and design: TK and KF. Acquisition of data: TK. Analysis and

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54 363 interpretation of data: TK. Drafting of the manuscript: TK. Critical revision of the

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57 364 manuscript for important intellectual content: MM and KF. Statistical analysis: YT and
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6 365 HO. Administrative, technical, or material support: YT and HO. Study supervision:
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9 366 MM and KF. Writing, reviewing, and editing: MM and KF.
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15 368 **Patients consent for publication:** Informed consent was obtained from all individual
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18 369 participants included in the study. Signed informed consent was obtained from each
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21 370 study participant prior to participation in the study.
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27 372 **Ethical approval:** The ethical aspects of this study were reviewed and approved by the
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30 373 institutional review board of Saga University Hospital (approval number: 2016-11-03).
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33 374 Written informed consent was obtained from all of the students and their guardians. All
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36 375 methods were carried out in accordance with relevant guidelines and regulations or
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39 376 Helsinki guidelines.
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45 378 **Provenance and peer review:** Not commissioned; externally peer-reviewed.
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51 380 **Date availability statement:** The datasets used and analyzed during the current study
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54 381 are available from the corresponding author on reasonable request.
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532 **Table 1. Background characteristics of junior high school students in the two**
 533 **groups**

		Control group (n = 79)	<i>H. pylori</i> group (n = 80)	<i>P</i> value
Sex	(male/female)	42/37	46/34	0.80
Age	(years)	14.73 ± 0.33	14.76 ± 0.32	0.71
BMI	(kg/m ²)	19.69 ± 3.48	19.67 ± 2.41	0.97
Delivery	(vaginal/C-section)	68/11	60/11	0.79
Nutrition	(breast/formula/mix)	37/6/36	27/15/36	0.07
School	(nursery/kindergarten/none)	25/54/0	53/25/2	<0.001
Allergies	(+/-)	5/75	7/73	0.55

534 Delivery: birth delivery style; C-section: cesarean section; nutrition: method of infant
 535 nutrition; school: pre-school situation; BMI: body mass index.

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6 **536 Figure legends**

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9 **537 Figure 1. Flowchart for *Helicobacter pylori* screening and treatment of junior high**
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12 **538 school students in Saga Prefecture and the selection method used to establish the**
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15 **539 two groups.**

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18 540 The *H. pylori* group comprised 80 students, consented to the study, tested positive for
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24 542 control group (n = 79) comprised those tested negative for both tests.

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30 **544 Figure 2. Alpha diversity of the 16S rRNA sequences in the control and *H. pylori***
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36 546 The control group exhibited lower microbial species richness compared with the
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39 547 *H. pylori* group. The observed species index (S. obs), chao 1 index, and
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42 548 abundance-based coverage estimator index all showed significantly higher diversity in
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45 549 the *H. pylori* group than in the control group ($*P < 0.001$). The Shannon index was not
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48 550 significantly different between the two groups ($P = 0.054$). OTUs: operational
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51 551 taxonomic units.

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7 **552 Figure 3. Beta diversity of the 16S rRNA/DNA/amplicon sequence data (control**
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9 **553 group vs *H. pylori* group)**

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12 554 PCO: principal coordinate analysis; PERMDISP: permutational analysis of multivariate
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15 555 dispersions.

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21 557 **Figure 4. The main 13 bacterial types present in the intestinal microbiota at the**
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24 558 **genus level, comparing the *H. pylori* and control groups.**

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27 559 **P* < 0.05; ***P* < 0.01.

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33 561 **Figure 5. The seven main bacterial types present in the intestinal microbiota at the**
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36 562 **genus level for the control group (A) and the *H. pylori* group (B) in association with**
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39 563 **body mass index (BMI)**

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42 564 **P* < 0.05; ***P* < 0.01. Low: BMI < 15; Mid: BMI of 15–25; High: BMI > 25.

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48 566 **Figure 6. Relative abundance of the *Prevotella* genus in relation to the BMI**
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51 567 **category in the *H. pylori* and control groups**

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54 568 Low: BMI < 15; Mid: BMI of 15–25; High: BMI > 25.

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57 569 **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

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571 **Figure 7. The ratio of the Firmicutes phylum to the Bacteroides phylum in relation**
572 **to the BMI category in the *H. pylori* and control groups**

573 Low: BMI < 15; Mid: BMI of 15–25; High: BMI > 25.

574 *** $P < 0.001$.

575

576 **Figure 8. Relative abundance of the *Subdoligranulum* genus in relation to the BMI**
577 **category in the *H. pylori* and control groups**

578 Low: BMI < 15; Mid: BMI of 15–25; High: BMI > 25.

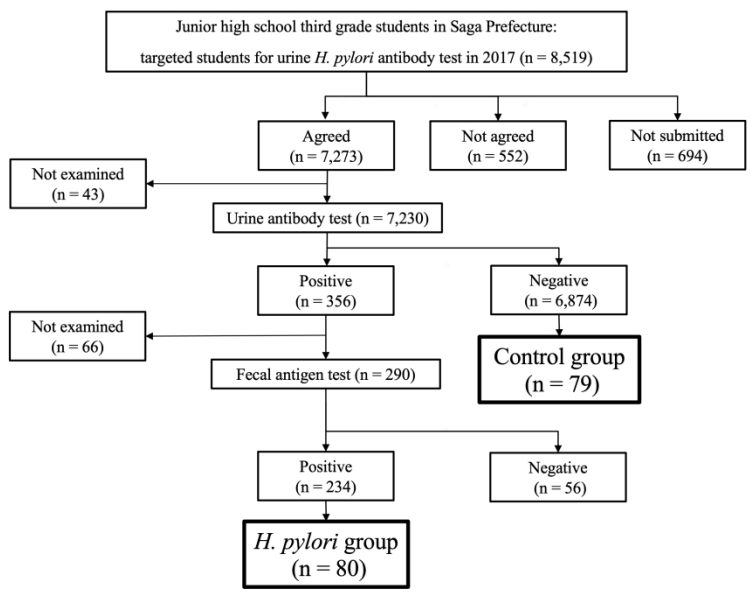
579 * $P < 0.05$; ** $P < 0.01$.

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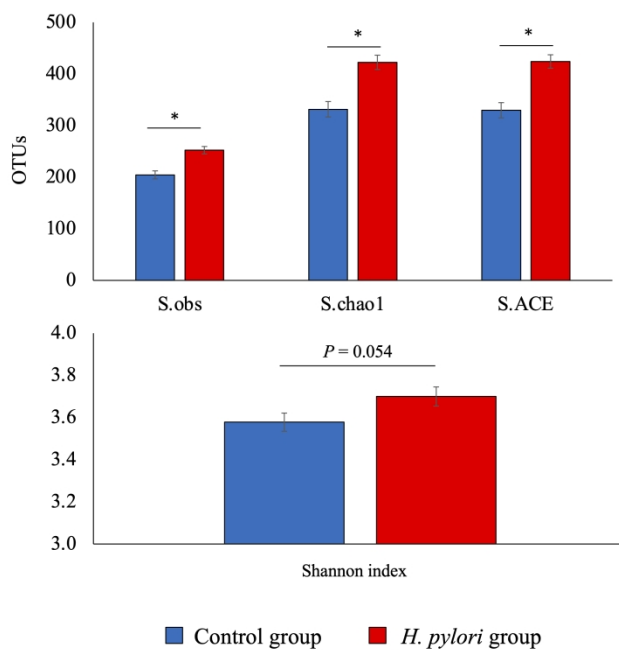
581 **Figure 9. Relative abundance of the *Collinsella* genus in relation to the BMI**
582 **category in the *H. pylori* and control groups**

583 Low: BMI < 15; Mid: BMI of 15–25; High: BMI > 25.

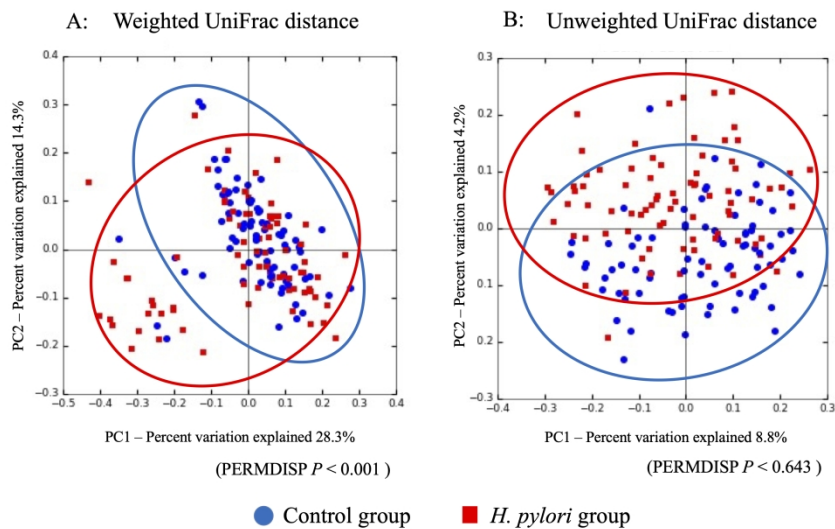
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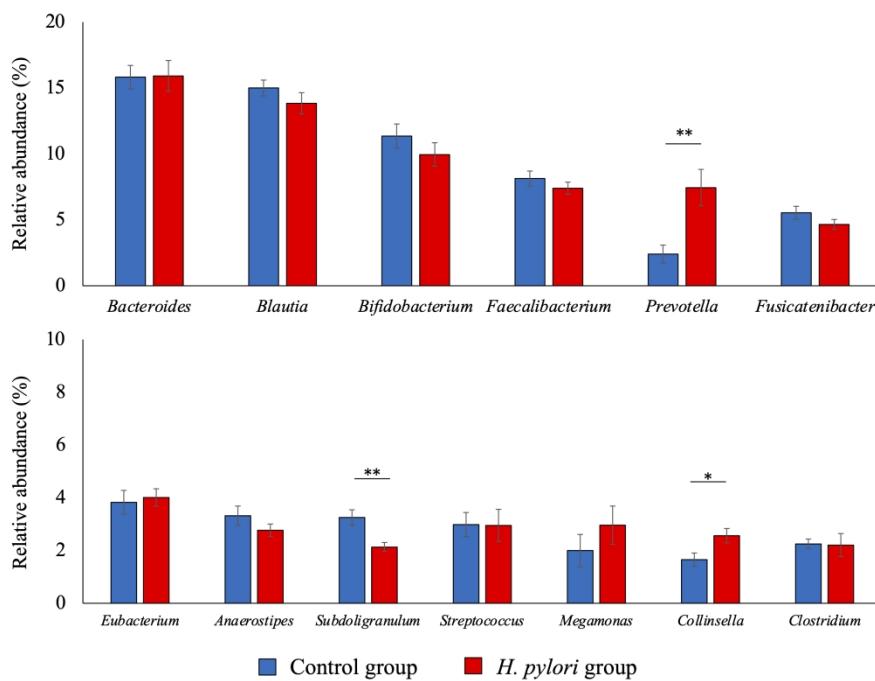


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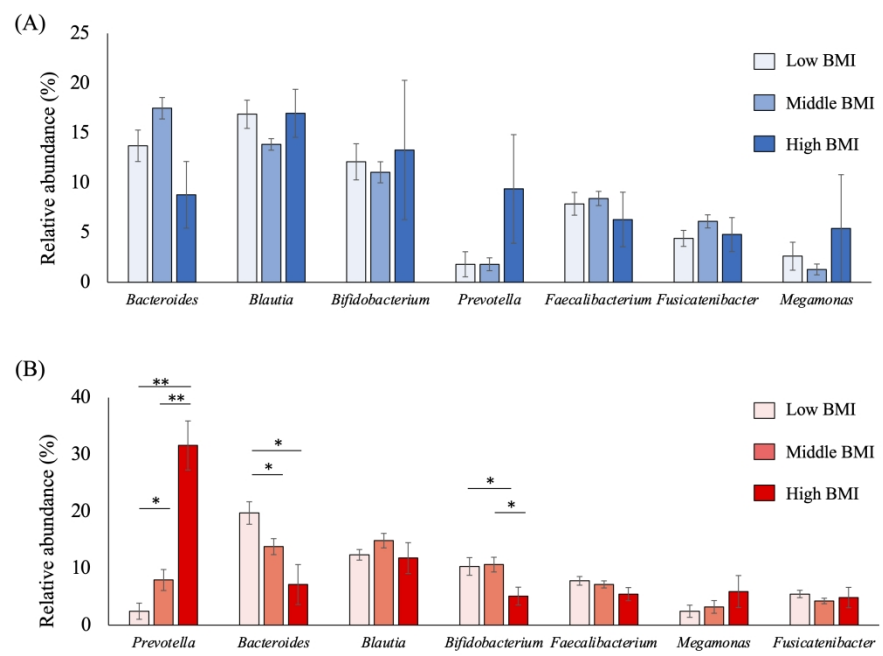
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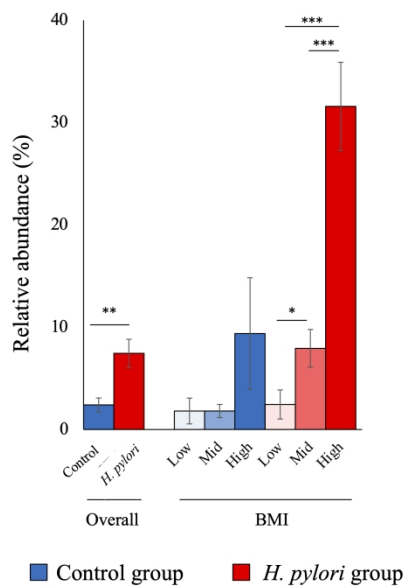


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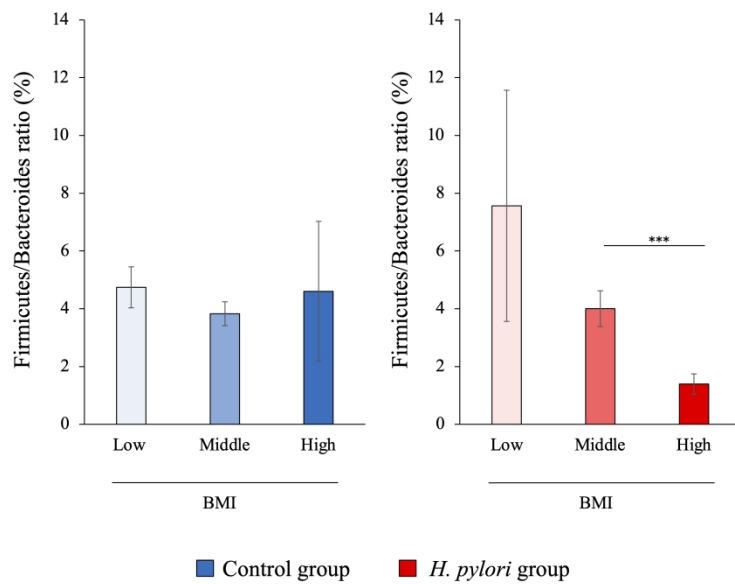
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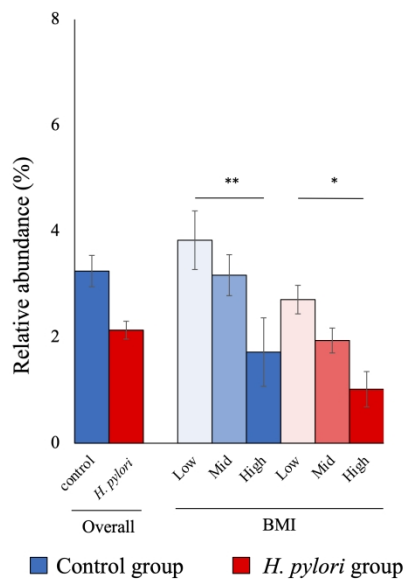


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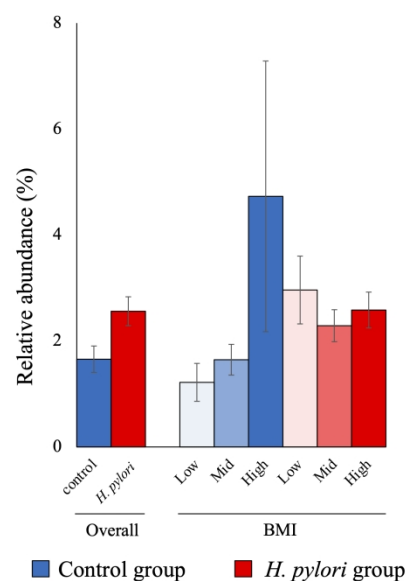
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Reporting checklist for cross sectional study.

Based on the STROBE cross sectional guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

Upload your completed checklist as an extra file when you submit to a journal.

In your methods section, say that you used the STROBE cross sectional reporting guidelines, and cite them as:

von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: guidelines for reporting observational studies.

			Page Number
Title and abstract			
Title	#1a	Indicate the study's design with a commonly used term in the title or the abstract	1
Abstract	#1b	Provide in the abstract an informative and balanced summary of what was done and what was found	2-3
Introduction			
Background / rationale	#2	Explain the scientific background and rationale for the investigation being reported	5
Objectives	#3	State specific objectives, including any prespecified hypotheses	5
Methods			
Study design	#4	Present key elements of study design early in the paper	5-6
Setting	#5	Describe the setting, locations, and relevant dates, including periods of	5-6

		recruitment, exposure, follow-up, and data collection	
1			
2	Eligibility criteria	#6a Give the eligibility criteria, and the sources and methods of selection of	6
3		participants.	
4			
5			
6		#7 Clearly define all outcomes, exposures, predictors, potential	6-9
7		confounders, and effect modifiers. Give diagnostic criteria, if applicable	
8			
9			
10	Data sources /	#8 For each variable of interest give sources of data and details of methods	6-9
11	measurement	of assessment (measurement). Describe comparability of assessment	
12		methods if there is more than one group. Give information separately	
13		for for exposed and unexposed groups if applicable.	
14			
15			
16	Bias	#9 Describe any efforts to address potential sources of bias	6-9
17			
18			
19	Study size	#10 Explain how the study size was arrived at	6-9
20			
21	Quantitative	#11 Explain how quantitative variables were handled in the analyses. If	6-9
22	variables	applicable, describe which groupings were chosen, and why	
23			
24			
25	Statistical	#12a Describe all statistical methods, including those used to control for	9
26	methods	confounding	
27			
28			
29	Statistical	#12b Describe any methods used to examine subgroups and interactions	9
30	methods		
31			
32			
33	Statistical	#12c Explain how missing data were addressed	9
34	methods		
35			
36			
37	Statistical	#12d If applicable, describe analytical methods taking account of sampling	9
38	methods	strategy	
39			
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41	Statistical	#12e Describe any sensitivity analyses	9
42	methods		
43			
44			
45	Results		
46			
47	Participants	#13a Report numbers of individuals at each stage of study—eg numbers	10
48		potentially eligible, examined for eligibility, confirmed eligible,	
49		included in the study, completing follow-up, and analysed. Give	
50		information separately for for exposed and unexposed groups if	
51		applicable.	
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55	Participants	#13b Give reasons for non-participation at each stage	10
56			
57	Participants	#13c Consider use of a flow diagram	10
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1	Descriptive data	#14a	Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders. Give information separately for exposed and unexposed groups if applicable.	10
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6	Descriptive data	#14b	Indicate number of participants with missing data for each variable of interest	10
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10	Outcome data	#15	Report numbers of outcome events or summary measures. Give information separately for exposed and unexposed groups if applicable.	10-12
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14	Main results	#16a	Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	10-12
15				
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19	Main results	#16b	Report category boundaries when continuous variables were categorized	10-12
20				
21	Main results	#16c	If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	10-12
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25	Other analyses	#17	Report other analyses done—e.g., analyses of subgroups and interactions, and sensitivity analyses	10-12
26				
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29	Discussion			
30				
31	Key results	#18	Summarise key results with reference to study objectives	13
32				
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34	Limitations	#19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias.	16
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39	Interpretation	#20	Give a cautious overall interpretation considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.	13-16
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44	Generalisability	#21	Discuss the generalisability (external validity) of the study results	13-16
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47	Other			
48	Information			
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51	Funding	#22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	18
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